



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US89/00876 <b>(22) International Filing Date:</b> 3 March 1989 (03.03.89) <b>(71) Applicant:</b> THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 2199 Addison Street, Berkeley, CA 94720 (US). <b>(72) Inventors:</b> GODENOW, Robert, S. ; 20 Ellen Court, Orinda, CA 94563 (US). OLSSON, Lennart ; 20A Ibstruvej, DK-2820 Gentofte (DK). <b>(74) Agent:</b> ROWLAND, Bertram, I.; Cooley Godward Castro Huddleson & Tatum, 5 Palo Alto Square, 4th Floor, Palo Alto, CA 94306 (US).		<b>(81) Designated States:</b> AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i> <i>With amended claims.</i>
<b>(54) Title:</b> CLASS I MHC MODULATION OF SURFACE RECEPTOR ACTIVITY  <b>(57) Abstract</b>  Methods and compositions are provided for regulating surface membrane receptor response by modulating the interaction between an MHC Class I antigen and the surface membrane receptor. Various techniques may be employed for enhancing or reducing the interaction between the Class I antigen and surface membrane receptor, e.g. enhancing production of the Class I antigen.		

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CLASS I MHC MODULATION OF SURFACE  
RECEPTOR ACTIVITY

5

INTRODUCTION

Technical Field

10           The field of the subject invention concerns modulation of surface membrane responses.

Background

15           The major histocompatibility complex (MHC) Class I antigens are expressed on virtually all types of vertebrate cells examined. These highly polymorphic transmembraneous glycoproteins have a 45 kD heavy chain consisting of a short cytoplasmic C-terminal tail, a transmembraneous region, and an extracellular N-terminal sequence which encompasses three domains,  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$ . The  $\alpha_1$ - and  $\alpha_2$ -domains carry all the immunological polymorphism, while the membrane-proximal  $\alpha_3$  is non-covalently associated with the 12 kD  $\beta_2$  microglobulin.

25           The MHC Class I antigen plays an essential role in restriction of the target cell repertoire of cytotoxic T-lymphocytes (CTL), which involves preferential utilization of the different polymorphic MHC Class I antigens, H-2K, -D or -L (for mouse) or HLA-A, -B, or -C (for human), e.g. in recognition of viral infected cells. For the most part, attention has been directed to the role of the MHC Class I antigens in restricting T-cell activity. However, some authors have suggested a broader role for the antigens, which will  
30  
35. be discussed below.

Relevant Literature

For a review of biological functions of MHC Class I antigens see Ohno, Immunol. Rev. (1977) 33:59-69; and Simonsen, Prog. Allergy (1985) 36:151-176. For  
5 a description of the insulin receptor see Cuatrecasas, Biol. Chem. (1972) 247:1980-1991; Kasuga et al., ibid. (1982) 257:10392-10399; and Kasuga et al., ibid. (1983) 258:10973-10980. For suggestion that Class I antigens and insulin receptors interact, see Olsson, In Cell  
10 Fusion: Gene Transfer and Transformation (eds. Beers & Bassett) 395-403 (Raven Press, New York, 1984); Simonsen and Olsson, Ann. Immunol. (1983) 134D:85-92. A relationship between receptors and their specific li-  
gands, where the reverse complement of limited regions  
15 in receptor mRNA are identified in the ligand DNA sequence is described by Bost et al., Proc. Natl. Acad. Sci. USA (1984) 82:1372-1375; and Bost et al., Biochem. Biophys. Res. Commun. (1985) 128:1373-1380. Other evidence supporting the interaction between MHC products  
20 and insulin receptor may be found in Fehlman et al., Proc. Natl. Acad. Sci. USA (1985) 82:8634-8637; Philips et al., ibid. (1986) 83:3474-3478; Due et al., ibid. (1986) 83:6007-6011, and Samson et al., J. Immunology (1986) 137:2293-2298. Suggestions of a correlation be-  
25 tween overexpression of certain Class I products and increased metastatic potential of particular tumors may be found in Wallich et al., Nature (1985) 315:301-305; Katzav et al., Int. J. Cancer (1984) 33:47-415; Olsson, Cancer Rev. (1986) 3:91-114; and Goodenow et al.,  
30 Science (1985) 230:777-783.

SUMMARY OF THE INVENTION

Methods and compositions are provided for  
modulating activity of cell surface receptors. The  
35 methods employ up or down regulation of cellular production of MHC Class I antigens or employ agonist or antagonist substances for non-covalent binding to the

cellular receptor or MHC Class I antigen to affect the complexation between the MHC Class I antigen and the cellular receptor or mimic the effect of the Class I antigen. The methods and compositions may be used in  
5 diagnosis and therapy.

#### DESCRIPTION OF SPECIFIC EMBODIMENTS

Methods and compositions are provided for modulating the response of cell surface receptors by varying the interaction between the cell surface receptor  
10 and MHC Class I antigen. The variation may be as a result of up or down regulation of Class I antigen production or concentration at the surface, providing agonists or antagonists for mimicking the non-covalent  
15 binding of the Class I antigen to the receptor or inhibiting such binding. Modulation of the Class I antigen-receptor interaction can be used in diagnosing and treating a large variety of conditions associated with cellular membrane receptors.

20 Human MHC Class I antigens are HLA-A, B, C, Qa and Tl. Of particular interest involved with the modulation of cellular receptors are the HLA-B and -C antigens, particularly the  $\alpha_1$ - and  $\alpha_2$ -domains, more particularly the  $\alpha_1$ -domain.

25 Of particular interest are the amino acid sequences involved in the polymorphic regions of  $\alpha_1$  and  $\alpha_2$ , ranging from amino acid 50 to amino acid 90, more particularly amino acids 55 to 90, usually 60 to 90, more particularly 65 to 90 or 90 to 120, more usually  
30 90 to 116, where the amino acid sequences of interest are usually in the C-terminus of the  $\alpha_1$ -domain and N-terminus of the  $\alpha_2$ -domain. The region 60-85, more particularly 65 to 85 or 70 to 85 are found to be of particular interest.

35 It is found that the amino acids from 83 to 85 may be of particular significance. For both MHC Class I D and K, or analogous HLA-B or C, the sequence is R Y

Y. Peptides of particular interest will comprise this sequence and may include at least about 20, usually at least about 15, and preferably not more than about 10 amino acids on either side of the sequence, preferably  
5 having at least 5 amino acids at the N-terminal side, and more preferably not having more than about 5-amino acids at the C-terminal side. The presence of two tyrosines is particularly desirable for the insulin receptor.

10 Desirably, the total number of amino acids will not exceed 20, preferably not exceed about 18, more preferably not exceed about 15 with the sequences indicated above.

Also of interest is the region from about  
15 amino acid 30 to amino acid 45, more particularly 32 to 40, particularly an oligopeptide of at least four amino acids, more usually at least about six amino acids, and preferably at least about eight amino acids, where the sequence includes a tetramer involving an acidic amino  
20 acid and a basic amino acid separated by one neutral amino acid, particularly a neutral amino acid of at least five carbon atoms and one of the acidic or basic amino acids is flanked by a neutral amino acid. Of particular interest is where the intervening neutral  
25 amino acid is an aromatic or aliphatic hydrocarbon amino acid, e.g. glycine or phenylalanine.

A large number of surface membrane proteins are involved with transduction of signals and serve as receptors for a wide variety of ligands. For the most  
30 part, receptors are defined by the ligand which activates the receptor for transduction or serves to endocytose the ligand. These receptors include endocrine, paracrine and autocrine receptors, adrenergic receptors, lipoprotein receptors, opiate receptors, and  
35 steroid receptors. These receptors include surface protein receptors for asialoglycoprotein, insulin, somatostatin, somatotropins; growth factors, such as

growth hormone, platelet derived growth factor, insulin like growth factor, epidermal growth factor,  $\alpha$ -transforming growth factor, nerve growth factor, fibroblast growth factor, somatomedin, vasopressin, 5 prostaglandins, eosinophil chemotactic factor, acetylcholine, thyroxine (TSH), epinephrine; endorphins, enkephalins and dynorphin; neurotensin, oxytocin, transferrin, substance P, lymphokines, such as 1-, 2-, 3-, and 4-, etc.; colony stimulating 10 factors, such as GM-CFS, M-CFS, E-CFS, etc.; lipoproteins, such as low density lipoprotein; steroids, such as estrogen, androgen, glucocorticoids, corticosteroids, etc. Of particular interest are receptors which are cycled, that is, internalized into 15 the cytoplasm and then returned to the plasma membrane surface. Illustrative of these receptors are the receptors for insulin, EGF, LDL, transferrin, interleukins, and asialoglycoprotein.

Modulation of the MHC Class I antigen activation can be achieved in a variety of ways. The number 20 of MHC antigen molecules at the surface can be increased or decreased by employing compounds which activate or inhibit the Class I antigen production. These compounds include interferon, dimethyl sulfoxide 25 (DMSO), tetradecylphorbol acetate (TPA), and retinoic acid. Alternatively, one may modulate the complex formation by employing antibodies to the  $\alpha_1$ - or  $\alpha_2$ -domain, particularly the  $\alpha_1$ -domain, which inhibit the interaction between the Class I antigen and the receptor. 30 Either polyclonal or monoclonal antibodies may be employed, particularly monoclonal. Alternatively, one may employ the monoclonal antibodies specific for the  $\alpha_1$ -domain to be used as immunogens for the production of anti-idiotypic antibodies, which will mimic the conformation of the Class I antigen epitope to which the 35 monoclonal antibody binds. Thus, the anti-idiotypic may act as a substitute Class I antigen and may serve to

block autoimmunity. The whole antibodies need not be employed, the variable region sufficing, or larger fragments such as Fab or  $F(ab')_2$ , Fab', etc.

5       The antibodies may be prepared in accordance  
with conventional ways. Particularly, the Class I an-  
tigen may be used as an immunogen and injected into an  
appropriate host, conveniently a mouse, for initiating  
an immune response. One or more booster injections may  
be employed at two or more week intervals. Two to  
10   three days after the last injection, the animal host  
may be sacrificed, the spleen isolated, and the B-lym-



substitutions.

Table A

Neutral		
Aliphatic		
5	Non-polar	
	small	G, A (P)
	large	V, I, L
	Polar	
	Oxy or Thio	S, T, C, M
10	Amide	N, Q
	Aromatic	F, W, H, Y
Charged		
	Acidic	D, E
15	Basic	K, R

( ) intends that the amino acid will normally not be used as a substitute for others on the same line.

20           It is found that the peptides which bind to the receptors enhance receptor activity. While not wishing to be bound to the theory, it appears that the peptides are involved with inhibiting internalization of the receptor. In this manner, the lifetime of the

25   ligand-receptor complex is extended, so that one observes an enhanced activity as a result of binding of ligand to the receptor. In addition, there may be other effects of the peptide, such as allosteric effects, which may enhance binding affinity, activation

30   effects, where the peptide results in activation of the receptor, so as to provide for transduction of a signal into the cytoplasm, or other effect, where the sum total of the result is an enhanced effect as compared to the absence of the peptide or MHC binding to the

35   receptor.

          In a variety of disease states, disease is as a result of a reduced presence of a particular receptor

at the surface or lower affinity for the ligand. In this situation, one could reduce the density of the Class I MHC antigen or provide for the peptide at an appropriate concentration, which allows for activation of the receptor. Conditions such as diabetes, Graves disease, arthritis, ankylosing spondylitis, Reiter's disease, pain, analgesia, viral disease, etc., could be associated with inadequate receptor response.

Alternatively, in other situations, one might wish to down regulate receptor binding, where one wishes to diminish the receptor response. Illustrative of such conditions is neoplasia, arthritis, lupus erythematosus, etc., where it is desirable to reduce the response to growth factors or other secreted factors which encourage proliferation or other undesirable response. In this situation, one could treat the target cells with a drug which would enhance the population of Class I antigens at the surface.

The subject peptides may affect one activity of the receptor differently from a different activity. For example, while with the insulin receptor, glucose uptake is enhanced, the tyrosine kinase activity is diminished. Thus, the subject peptides may selectively modify a receptor having a plurality of activities.

Instead of a change in the MHC Class I antigen population at the surface, the effective concentration of Class I antigen for complexing with receptors may be reduced. It is noted that viral infections deplete Class I antigens at the surface and in appropriate situations may be used for this purpose. As already indicated, Class I antigen depletion could also be achieved using antibodies or oligopeptides which bind to the Class I antigen at or near the complexing site inhibiting complexation with the receptor or bind to the receptor at or near the complexing site inhibiting complexation with the Class I antigen. These compounds can be prepared by employing sequences comparable to

polymorphic sequences, particularly in the  $\alpha_1$ -domain of the Class I antigen, more particularly HLA-B or -C antigens (mouse H-2L or D).

Of particular interest are oligopeptides comprising at least a portion of one of the following sequences, where the oligopeptides comprise as the active sequence, at least six amino acids, usually at least eight amino acids, more usually at least 12 amino acids, and fewer than 40 amino acids, more usually fewer than 30 amino acids, preferably, not more than about 25 amino acids, preferably being from about 8 to 25 amino acids, more preferably about 8 to 20 amino acids. It is understood that up to five, more usually up to about three substitutions or deletions may be made in the subject sequences, where the change will not be more than about 20 number %, usually not more than about 10 number % of the number of amino acids in the active sequence. Also the following sequences may be joined together either contiguously or by bridges of not more than about 20 amino acids, more usually not more than about 10 amino acids. Furthermore, where the sequences overlap, it is intended that the overlapping sequences not be repeated, but rather the non-overlapping sequences joined in proper sequence.

The oligopeptide will have at least six amino acids which are the same or substantially the same as a sequence included in the following sequence.

1. D T aa<sup>32</sup> F V R F D S D aa<sup>40</sup> aa<sup>41</sup>
  2. F V R F D S D aa<sup>40</sup> aa<sup>41</sup> S P R aa<sup>45</sup>
  3. W aa<sup>52</sup> E Q aa<sup>55</sup> aa<sup>56</sup> G P E Y W
  4. W aa<sup>61</sup> aa<sup>62</sup> aa<sup>63</sup> T aa<sup>65</sup> aa<sup>66</sup> aa<sup>67</sup> K aa<sup>69</sup> aa<sup>70</sup> aa<sup>71</sup> Q
  5. W aa<sup>61</sup> aa<sup>62</sup> aa<sup>63</sup> aa<sup>64</sup> aa<sup>65</sup> aa<sup>66</sup> aa<sup>67</sup> K aa<sup>69</sup> aa<sup>70</sup>  
aa<sup>71</sup> aa<sup>72</sup> aa<sup>73</sup> aa<sup>74</sup> aa<sup>75</sup> aa<sup>76</sup> aa<sup>77</sup> aa<sup>78</sup> aa<sup>79</sup> aa<sup>80</sup>  
aa<sup>81</sup> aa<sup>82</sup> aa<sup>83</sup> aa<sup>84</sup> aa<sup>85</sup>
  6. E Q aa<sup>73</sup> aa<sup>74</sup> R V aa<sup>77</sup> aa<sup>78</sup> R aa<sup>80</sup> aa<sup>81</sup> aa<sup>82</sup> R Y Y
- wherein:

aa<sup>32</sup> is any neutral aliphatic amino acid of from 4 to 6 carbon atoms particularly N, Q, V, I, or L, more particularly Q or L;

5 aa<sup>40</sup> is an aliphatic amino acid, charged or uncharged, usually non-polar or acidic of from 2 to 5, more usually 2 to 4 carbon atoms, particularly G, A, P, D or E, more particularly A or D;

10 aa<sup>41</sup> is an aliphatic amino acid, charged or uncharged of from 2 to 5, usually 3 to 5 carbon atoms, particularly G, A, P, S, T, D or E, more particularly A, T and E;

aa<sup>44</sup> is P, N, or Q, particularly P or Q;

aa<sup>45</sup> is any aliphatic amino acid, particularly G, A, S, T, M, K, R, or E, particularly G, E, or K;

15 aa<sup>52</sup> is a neutral aliphatic amino acid of from 4 to 6 carbon atoms, particularly V, I, L or M, more particularly V or I;

aa<sup>55</sup> is any charged amino acid, particularly K, R, D, or E, more particularly K or E;

20 aa<sup>56</sup> is a charged amino acid, particularly D, E, K or R, more particularly E or K;

aa<sup>61</sup> is D or E;

aa<sup>62</sup> is K, R, G, or A, particularly R or G;

25 aa<sup>63</sup> is any aliphatic amino acid other than basic of from 4 to 6 carbon atoms, particularly D, E, I, L, V, N, or Q, more particularly E, N, or Q;

aa<sup>64</sup> is S, T, or M, particularly T;

30 aa<sup>65</sup> is any polar or basic amino acid of 4 to 6 carbon atoms, particularly N, Q, K or R, more particularly Q;

aa<sup>66</sup> is any aliphatic amino acid of from 4 to 6 carbon atoms, particularly L, I, V, K, R, N, or Q, more particularly K, I or N;

35 aa<sup>67</sup> is any neutral aliphatic or aromatic amino acid, particularly G, A, L, V, I, S, T, M, C F, Y, N, or Q, more particularly C, S, Y, or M;

aa<sup>68</sup> is K or R, particularly K;

aa<sup>69</sup> is any aliphatic neutral or acidic amino acid, particularly D, E, G, A, S, T, or M, particularly A or T;

5 aa<sup>70</sup> is any aliphatic amino acid, neutral, polar, or basic (other than acidic) from 3 to 6, usually 4 to 6 carbon atoms, particularly N, Q, K, R, S, or T, more particularly N, Q, or K;

10 aa<sup>71</sup> is any aliphatic amino acid other than basic, usually from 2 to 5 carbon atoms, particularly G, A, S, T, D, or E, more particularly A or T;

aa<sup>72</sup> is N or Q, particularly Q;

aa<sup>73</sup> is S, T, F, Y, H, or W, particularly T;

aa<sup>74</sup> is D, E, F, Y, H, or W, particularly Y or D;

15 aa<sup>75</sup> is K or R, particularly R;

aa<sup>76</sup> is an aliphatic amino acid other than basic of from 4 to 6 carbon atoms, particularly D, E, V, I, or L, more particularly E or V;

20 aa<sup>77</sup> is a polar aliphatic amino acid of from 3 to 6 carbon atoms particularly N, Q, S, T, D, or E, more particularly N, D or S;

aa<sup>78</sup> is a non-polar aliphatic amino acid of from 3 to 6 carbon atoms, particularly A, P, V, I, or L, more particularly L;

25 aa<sup>79</sup> is K or R, particularly R;

aa<sup>80</sup> is a neutral aliphatic amino acid of from 3 to 6, usually 4 to 6 carbon atoms, particularly S, T, N, Q, I, V, or L, more particularly N, T, or I;

30 aa<sup>81</sup> is an aliphatic non-polar amino acid, particularly G, A, L, I, or V, more particularly A or L;

35 aa<sup>82</sup> is an aliphatic amino acid other than acidic, of from 2 to 6, usually 5 to 6, carbon atoms, particularly K, R, G, A, L, I, or V, more particularly L or R;

aa<sup>83</sup> is an aliphatic amino acid other than acidic of from 2 to 6 carbon atoms, particularly K, R,

G, A, L, I, or V, more particularly G or R;

aa<sup>84-85</sup> are aromatic amino acids, particularly F, Y, H, or W, more particularly Y.

Preferably, there will usually not be more than three mutations in the above sequence as substitutions, deletions, or insertions.

Of particular interest is an amino acid sequence of at least 6, usually at least 8, amino acids coming within the following sequence.

10

W D/E R aa<sup>63</sup> T Q/R aa<sup>66</sup> aa<sup>67</sup> K aa<sup>69</sup> aa<sup>70</sup> aa<sup>71</sup>  
Q T/W aa<sup>74</sup> R V/E aa<sup>77</sup> L R aa<sup>80</sup> L/A L/R G/R Y Y

wherein:

15 aa<sup>63</sup> is E, I, or N;  
aa<sup>66</sup> is I, N, or K, particularly I;  
aa<sup>67</sup> is A, C, S, M, or Y, particularly Y or C;  
aa<sup>69</sup> is G, A, T, or P, particularly A or T;  
aa<sup>70</sup> is Q, N, or K;  
20 aa<sup>71</sup> is A, E, or T;  
aa<sup>74</sup> is D, F, or Y, particularly D or Y;  
aa<sup>77</sup> is N, S, or D;  
aa<sup>80</sup> is I, N, or T.

where when two amino acids are indicated at a particular site, either amino acid may be employed interchangeably. Up to three of the amino acids may be subject to conservative or non-conservative changes, there being from 0 to 2 deletions or insertions of from 1 to 2 amino acids.

30 The oligopeptides may be provided in a variety of ways, being joined to non-wild-type flanking regions, as fused proteins, joined by linking groups or directly covalently linked through cystine (disulfide) or peptide linkages. The oligopeptides may be joined  
35 to a single amino acid at the N- or C-terminus or a chain of amino acids. The fused peptides may be extended to provide convenient linking sites, e.g. cys-

teine or lysine, to enhance stability, to bind to particular receptors, ease of purification, change the physical characteristics, e.g. solubility, charge, etc, stabilize the conformation, etc. The oligopeptide may  
5 be N- or C-terminal or internal.

The oligopeptide may be joined to other peptides for a variety of purposes. The oligopeptide may be linked through a variety of bifunctional agents, such as maleimidobenzoic, methylthioacetic acid, mercaptobenzoic acid, S-pyridyl dithiopropionate, etc.  
10 The oligopeptides may be linked to proteins to provide immunogens for the production of antibodies. The oligopeptides may be linked, particularly by an intracellular cleavable linkage, to antibodies for site directed action.  
15 The oligopeptides may be linked to peptides to enhance stability, for site directed action, to provide additional physiological activity or the like. For conjugation techniques, see, for example, U.S. Patent Nos. 3,817,837; 3,853,914; 3,850,752;  
20 3,905,654; 4,156,081; 4,069,105; and 4,043,989, which are incorporated herein by reference.

The oligopeptides may also be modified by incorporation into the lumen of vesicles, e.g. liposomes, which in turn may be bound to ligands or receptors for  
25 direction to particular cells or tissue.

The oligopeptides may be used as ligands to determine the presence of particular receptors as a diagnostic. Thus, cells could be screened, intact or as a lysate, for the population of one or more receptors which bind the oligopeptide. The oligopeptides  
30 could be labeled, directly or indirectly, with a label which provides a detectable signal, e.g. radioisotope, fluorescers, enzyme, particle, chemiluminescer, etc. Thus, cells from tissue, e.g. biopsies, blood, or the  
35 like may be diagnosed in vitro or in vivo for the presence of receptors binding to the oligopeptides. In addition, the binding pattern with the MHC Class I

antigen of various cells can be determined. Diseased states as a result of inadequate complexation between MHC Class I antigens and receptors can be determined. A large number of protocols are known and have been developed and appear in the patent and scientific literature. Commercially available assays include ELISA, EMIT, SLFIA, RIA, etc.

The oligopeptides may be employed in a variety of ways. For therapy, they may be administered parenterally, e.g. by injection at a particular site, for example, subcutaneously, intraperitoneally, intravascularly, or the like.

The formulations will usually involve a physiologically acceptable medium, such as deionized water, saline, aqueous ethanol, ethanol, phosphate buffered saline, and the like. Other additives may be included, such as buffers, stabilizers, other proteins, bacteriocides, or the like. The manner of formulation will vary depending upon the purpose of the formulation, the particular mode employed for modulating the receptor activity, the intended treatment, and the like. The formulation may involve capsules, liposomes, time delayed coatings, pills, or be formulated in pumps for continuous administration. Because of the wide variety of modes of treatment, the varying responses, the different disease states, or the like, no useful limits may be given for the concentration of the active components. These can be determined empirically in accordance with known ways. See, for example Harrison's, Principles of Internal Medicine, 11th ed. Braunwald et al. ed, McGraw Hill Book Co., New York, 1987.

The oligopeptides of this invention may be prepared in accordance with conventional techniques, such as synthesis, recombinant techniques, or the like. See, for example, Maniatis et al., Molecular Cloning: a laboratory manual, CSH Laboratory, Cold Spring Harbor, N w York, 1982; use of a Beckman Model 990 peptide



synthesizer or other commercial synthesizer.

The following examples are offered by way of illustration and not by way of limitation.

5

#### EXPERIMENTAL

Mutant line R1E, derived from the murine thymoma line R1 by chemical mutagenesis (Parns and Seidman, Cell (1982) 29:661-669; Allen et al., Proc. Natl. Acad. Sci. USA (1986) 83:7447-7451) expresses in contrast to R1, none of the parental H2<sup>k</sup> haplotype antigens of the C58 strain of origin due to the lesions induced in the  $\beta_2$ -microglobulin ( $\beta_2m$ ) gene in R1E. Specific insulin binding to R1E murine thymoma cells and various R1E transfectants was determined, with the binding being performed as described in Gavin et al., J. Biol. Chem. (1973) 248:2202-2207 and Due et al., Diabetologia (1985) 28:749-755.

All cells were cultivated in RPMI-1640 with 15% fetal calf serum (FCS) and with the various additives as indicated (Allen et al., supra (1986)). Prior to insulin binding assays, the cells were seeded in RPMI-1640 with 10% FCS and at a density of  $2 \times 10^4$  cells/ml, harvested three days later, viability assured to be >95% by trypan dye exclusion, and the cells subsequently resuspended in assay buffer for insulin binding at a concentration of  $7.5 \times 10^7$  cells/ml. <sup>125</sup>I-labeled human insulin in a final concentration of 50 pM (labeled in the A14 position and obtained from NOVO A/S, Denmark) was added and the cells incubated for 90 minutes at 18°C in a shaking waterbath. Two ml ice-cold assay buffer were added at the end of incubation, the cells centrifuged at 300 g for 5 minutes, at 100 g for 10 minutes, and the amount of <sup>125</sup>I-insulin in the pellet counted in a  $\gamma$ -counter. Non-specific binding was estimated as the amount of <sup>125</sup>I-insulin binding in the presence of  $10^{-6}$ M unlabeled insulin, and specific insulin binding calculated as the difference in binding

of  $^{125}\text{I}$ -insulin with and without unlabeled insulin. Specific binding <1% was estimated to be non-specific considering the Scatchard plots and the specific binding as related to cell number.

5           Scatchard plots were done for lines R1, R1E, R1E/ $\beta 2\text{m}$ , R1E/D<sup>b</sup>, R1E/ $\beta 2\text{m}/\text{K}^b$ , R1E/ $\beta 2\text{m}/\text{D}^b$ , R1E/ $\beta 2\text{m}/\text{D}^b\Delta$ , and R1E/ $\beta 2\text{m}/\text{D}^b$  - (1 + 2) with  $3 \times 10^7$  cells per sample and each point representing duplicate or triplicate samples. The Scatchard plots were repeated 3-10 times  
10           for each determination. Only R1 and R1E/ $\beta 2\text{m}/\text{D}^b$  displayed applicable amounts of insulin receptor (IR). The curve observed shows that in addition to high affinity IR, these cells also have appreciable amounts of receptors with lower affinity for insulin, which may to  
15           some extent be due to indirect effects of transfection and/or co-expression of other insulin binding receptors such as those for IGF-I (Rechler and Hessley In Poly-peptide Hormone Receptors (ed B.I. Posner) pp 227-297, Marcel Dekker, New York (1985)).

20           The R1 murine thymoma cells have a cell surface density of IR comparable to other lymphocyte cell populations in contrast to the human IM-9 cell line often used for insulin assays, and which is an Epstein-Barr virus transformed line with exceptionally high  
25           amounts of non-functional IR. It was accordingly necessary in the R1/R1E system to use comparatively high amounts of cells per sample. Titration of specific insulin binding as related to cell number demonstrated that the optimal cell number per sample for specific  
30           insulin binding was  $7 \times 10^7$  cells - impractical to use on all Scatchard plots. The curves for R1 and R1E/ $\beta 2\text{m}/\text{K}^b$  show that these two lines did not express significant amounts of IR.

35           Insulin receptor mRNA in R1, R1E and R1E transfectants was determined as follows. Total RNA was isolated from cells as per Chirgwin et al., Biochemistry (1979) 18:5294-5299, and poly A+ RNA selected as

per Maniatis et al., Molecular Cloning 1A Laboratory Manual CSH Laboratory, Cold Spring Harbor, New York, (1982). For Northern blot analysis, 5 µg of poly A+ selected murine liver mRNA was fractionated on a 1.0% agarose-formaldehyde gel (Church and Gilbert, Proc. Natl. Acad. Sci. USA (1984) 81:1991-1995) and blotted on a Zeta-probe nylon membrane (Bio Rad Laboratories, Richmond, CA). Insulin receptor-specific sequences were detected by hybridization with a synthetic DNA oligonucleotide representing amino acids 732-741 inferred from the insulin receptor cDNA precursor (Ullrich, et al., Nature (1985) 313:756-761). Hybridization and washing conditions were as per Church and Gilbert, supra (1984), except that hybridization and washes were at 45°C. Approximately 1 µg and 1:10 dilution of poly A+ selected mRNA from mouse liver and appropriate cell lines was spotted on the Zeta-Probe membranes and hybridized as above. Molecular weight markers for Northern blot analysis were purchased from Bethesda Research Laboratories (Bethesda, MD). A predominant species of 4.8 kb from mouse liver hybridized to the human insulin receptor oligonucleotide. This species was also noted by Ullrich et al., supra (1985), in human placental mRNA with radiolabeled cloned human insulin receptor cDNA sequences.

Reverse complementarity between D<sup>k</sup><sub>α1</sub> and insulin receptor was determined as follows. The reverse (3'-5') complement of the murine MHC Class I D<sup>k</sup> and K<sup>k</sup> genes were screened for nucleic acid and protein sequence using DNASIS (Hitachi Corp., Japan, and Intelligenetics, Palo Alto, CA) computer programs. 75% amino acid homology was noted between the reverse complement of K<sup>k</sup><sub>α1</sub> aa 34-41 and the human insulin receptor signal peptide aa 14-22. All noted homologies between D<sup>k</sup> and K<sup>k</sup> and human insulin, estrogen, epidermal growth factor, or interleukin-2 receptors were considerably lower (<50%). The degree of homology between D<sup>k</sup><sub>α1</sub> and IR is

within the limits of homology considered significant by  
 Bost et al., Biochem. Biophys. Res. Commun. (1985)  
128:1373-1380.

5

Figure 3

	N term									C term	
D <sup>k</sup> aa 34-41	---	Arg	Phe	Asp	Ser	Asp	Ala	Glu	Asn	---	
10	5'									3'	
D <sup>k</sup> nt 100-125	---	GCGC	TTC	GAC	AGC	GAC	GCG	GAG	AAT	---	
15	3'									5'	
D <sup>k</sup> complement	---	CGCG	AAG	CTG	TCG	CTG	CGC	CTC	TTA	---	
	Deduced peptide										
	from reverse	Arg	Glu	Ala	Val	Ala	Ala	Pro	Leu		
20	(3'-5')	.	.	.	.	.	.	.	.		
	complement	.	.	.	.	.	.	.	.		
	Insulin receptor	---	Arg	Gly	Ala	Ala	Ala	Ala	Pro	Leu	---
	signal sequence										
25	aa-22 - -14										

The two amino acids neglecting to show exact  
 homology between the reverse complement of D<sup>k</sup>al and the  
 human IR are mismatches in the third base codon. Exact  
 30 homology is represented by two ., whereas third base  
 mismatch is represented by one .. By scanning 4000  
 available sequences in the Bionet gene bank, no signi-  
 ficant homology was detected between the deduced pep-  
 tide or its DNA equivalent.

35. The surface proteins of the various cell lines  
 were screened using fluorescent labeled monoclonal  
 antibodies and a FACS.

**Table 1**  
**Fluorescence-Activated Cell Sorter (FACS) Analysis**  
**R1, R1E and the Transfectants for Expression of H-2 and  $\beta 2m$**

C 11 Lines <sup>c</sup>	H-2 specificity monoclonal antibodies <sup>a</sup>	K <sup>k</sup> D <sup>k</sup> (28-8-6)	K <sup>k</sup> (11.4)	K <sup>b</sup> (20-8-4)	D <sup>k</sup> (15-5-5)	D <sup>b</sup> (28-14-8)	β2m <sup>b</sup>	β2m
R1		750	505	<10	435	16	<10	175
R1E		25	<10	<10	<10	<10	<10	<10
R1E/β2m		290	80	15	90	15	225	80
R1E/D <sup>b</sup>		145	30	<10	30	570	<10	<10
R1E/β2m/D <sup>b</sup>		370	115	10	170	1240	450	390
R1E/β2m/K <sup>b</sup>		400	215	410	260	<10	510	425
R1E/β2m/DA		345	170	10	320	250	200	105
R1E/D <sup>b</sup> -(1+2)		190	70	<10	50	825	<10	<10
R1E/β2m/D <sup>b</sup> -(1+2)		350	105	10	130	880	210	160

Legend to Table 1

<sup>a</sup> The monoclonal antibodies have been described previously (Ozato et al. Transplantation (1982) 34:113-120). For staining,  $10^6$  cells were incubated with  $1 \mu\text{g}$  anti-body/ml at  $4^\circ\text{C}$  for 1 hour, washed twice in phosphate buffered saline (PBS), and incubated for another hour at  $4^\circ\text{C}$  with fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse polyclonal antibody (purchased from DAKO, Denmark). The cells were washed twice in PBS and analyzed. Cells incubated with FITC-conjugated secondary antibody served as negative controls. A shift of  $>15$  channel numbers on the linear fluorescence scale was considered significant; all samples were analyzed both on a logarithmic and linear fluorescence scale. The absolute number of bound FITC molecules per cell was estimated as described (Due et al., supra, (1985)). It should be noted that comparison of FACS data to estimate the relative proportion of different H-2 molecules only is reasonable, when the same primary antibody is used.

<sup>b</sup> Expression of H-2 was for all lines examined both by solid phase radioimmunoassay (RIA) with  $^{125}\text{I}$ -labeled protein A as secondary reagent and by FACS as described above. Briefly, RIA assays (Weiss et al., Nature (1984) 310:650-655) were done by plating  $5 \times 10^5$  cells (96 well microtiterplate) in  $50 \mu\text{l}$  Eagle's minimum essential medium (MEM) + 10% FCS.  $50 \mu\text{l}$  diluted anti-body in MEM + 10% FCS was then added. The cells were incubated 4 hours at  $4^\circ\text{C}$ , pelleted and washed with MEM + 10% FCS.  $^{125}\text{I}$ -Protein A was added (Amersham) to 100,000 cpm and incubated for 16 hours at  $4^\circ\text{C}$ . Cells were pelleted and washed three times with MEM + 10% FCS before counting in a Beckman Gamma Counter. All samples were done in duplicate and with less than 5% variation on all samples counted.

<sup>c</sup> The R1, R1E lines, the H-2K and H-2D genes, and the procedures for transfection have been described in detail previously (Allen *et al.*, Proc. Natl. Acad. Sci. USA (1986) 83:7447-7451; Goodenow *et al.*, Science (1982) 215:677-679). The various designations indicate: R1E/ $\beta$ 2m, transfected with  $\beta$ 2<sup>b</sup>; R1E/K<sup>b</sup>; transfected with K<sup>b</sup>; R1E/ $\beta$ 2m/K<sup>b</sup>, transfected with  $\beta$ 2m and K<sup>b</sup>; R1E/ $\beta$ 2m/D<sup>b</sup>, transfected with  $\beta$ 2m and D<sup>b</sup>, but the D<sup>b</sup> cell surface antigen was down-regulated with monoclonal antibody to D<sup>b</sup>; R1E/ $\beta$ 2m/D<sup>b</sup>-(1+2), transfected with  $\beta$ 2m and truncated D<sup>b</sup> gene, only expressing the  $\alpha$ 3-domain on the cell surface; R1E/D<sup>b</sup>-(1+2), transfected with truncated D<sup>b</sup>, only expressing the  $\alpha$ 3-domain.

Adenocarcinoma cell line LT85 (Callahan *et al.*, J. Immunol. (1983) 471-474) lacks appreciable cell surface expression of the H-2 antigens characteristic of the strain of derivation. Interferon- $\alpha$  (IFN- $\alpha$ ) treatment significantly increases the cell surface density of both H-2K<sup>k</sup>, H-2D<sup>k</sup> and increases in parallel the specific binding of insulin. Alternatively, the ultraviolet light-induced fibrosarcoma LR335 (Daynes *et al.*, Transplantation (1977) 23:343-348), unlike R1E or LT85, expresses appreciable levels of the H-2K<sup>k</sup> antigen, endogenous to the strain of origin with negligible H-2D<sup>k</sup>, unless induced with IFN. As shown in Table 2, IFN treatment markedly increases both H-2D<sup>k</sup> expression and the binding of insulin, approaching the levels of the other positive cell lines tested. These results support the conclusion drawn from work on the R1E transfectants: the control of IR expression maps to the end of the MHC D region.

Table 2

Effect of Interferon on MHC Class I and  
Insulin Receptor Expression

Cell Line	Interferon Treatment	H-2 allele Expression <sup>a</sup>		Specific Insulin Binding (%)
		K <sup>k</sup> (16-1-11)	D <sup>k</sup> (15-5-5)	
LT85	None	3,370	1,030	2.0
	+	19,170	6,500	3.2
LR 335	None	16,230	1,000	<1.0
	+	28,770	6,639	2.1

<sup>a</sup> Estimated by radioimmunoassay as described in Table 1 and the results given as cpm/5 x 10<sup>5</sup> cells

<sup>b</sup> The interferon treatment was done with alpha-interferon.

To map the control of insulin receptor in a third haplotype, several Class I variants of the BALB/c S49 thymoma line (Joseph *et al.*, *J. Immunol.* (1986) 137:4016-4020) were tested for insulin binding. As shown in Table 3, the H-2K/H-2D positive variant displayed significant hormone binding relative to the H-2K minus variant, suggesting that the D molecules support the cell surface expression of the receptor. The homology shared between H-2D<sup>b</sup> and H-2L<sup>d</sup> suggest that the H-2L locus also exerts an interaction with the IR.



Table 3

Expression of MHC Class I and Insulin Receptor  
in Six Murine Cell Lines of Two Different Haplotypes

5	MHC Class I Expression <sup>b</sup>	Cell lines <sup>a</sup>					
		3LL/G2	3LL/G4	28	33	36	29
10	K <sup>b</sup> (20-8-4)	14,840	2,500	-	-	-	-
	D <sup>b</sup> (28-14-8)	3,130	7,440	-	-	-	-
15	K <sup>d</sup> (20-8-4)	-	-	3,572	1,124	940	292
	D <sup>d</sup> (34-1-2)	-	-	4,930	661	278	280
	L <sup>d</sup> (28-14-8)	-	-	559	297	261	341
20	K <sup>k</sup> (negative control;11.4)	-	-	254	297	286	595
Specific Insulin Binding (%)		2.4	5.0	3.1	1.8	<1.0	<1.0

25

<sup>a</sup> All cell lines have been described elsewhere (Olsson and Forehammer, Proc. Natl. Acad. Sci. USA (1984) 81:3389-3393; Joseph et al., J. Immunol. (1986) 137:4016-4020). The lines numbered 28, 33, 36, and 29 have previously been designated S49.1, S49.2, S49.3, and S49.4, respectively.

30

<sup>b</sup> Estimated by RIA (see Table 1) with monoclonal antibodies as indicated and the data given as cpm/5 x 10<sup>5</sup> cells.

35.

Study of MHC Class I PeptideMATERIAL AND METHODS5 Cell Culture

All cell lines are grown in minimal essential medium (MEM, Gibco) supplemented with 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 1.0% non-essential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum (Hyclone) excepting R1E and R1E transfectants which are grown in RPMI (Gibco) and supplemented as above. The following cell lines are used in this study: P815- a murine mastocytoma of H-2<sup>d</sup> origin; R1E and related transfectants- murine lymphomas of H-2<sup>k</sup> origin but transfected with H-2<sup>b</sup> genes (Allen et al., (1986)); human lymphoblastoid lines #1 and #2- Epstein-Barr virus transformed lymphocytes from two related individuals, #2 being a Type I diabetic; LR335- a UV induced murine fibrosarcoma of H-2<sup>k</sup> origin.

20

Hormone Binding

Hormone binding assays are based on descriptions by Freychet Diabetologia (1976) 12(2):85-100; and Freychet et al., Endocrinology (1977) 100:115-121 with modifications. All reactions are carried out in 200 µl volumes in 96-well, round-bottomed microtiter plates (Dynatech Labs, VA) in MEM (Gibco) with 0.5% bovine serum albumin (Sigma fraction V) and 0.1% sodium azide to prevent internalization and subsequent degradation of membrane proteins. Sodium azide at 0.1% does not alter insulin binding. Cells are resuspended at  $1 \times 10^7$  to  $1 \times 10^8$ , depending on the cell type and, when appropriate, pre-incubated at 4° for one hour in the presence of peptide at 100 µg/ml. Specific insulin binding is measured using <sup>125</sup>I-insulin (Amersham, 2000 Ci/mmol, 370 kBq/10 uCi) as tracer at 50-100 pM. Incubation is carried out for 90 minutes at 20°. Cells

are then washed three times in ice-cold MEM to remove unbound insulin and the pellets resuspended to 100  $\mu$ l and counted in a Beckman gamma counter. Non-specific insulin binding is measured in the presence of 100  $\mu$ g/ml unlabeled porcine insulin (Sigma). Specific EGF binding is measured in a similar fashion using  $^{125}$ I-EGF (Amersham, 150  $\mu$ Ci/mg) as tracer and unlabeled mouse submaxillary gland EGF (Sigma) at 1  $\mu$ g/ml is used as cold competitor.

10

### RESULTS

#### Enhanced Insulin Binding Mediated by an MHC Class I Peptide

To investigate H-2/IR interactions on the cell surface, a peptide was synthesized corresponding to the  $\alpha_1$  domain, aa<sup>61-85</sup>, of the H-2L<sup>d</sup> protein. The sequence is E-R-I-T-Q-I-A-K-G-Q-E-Q-W-F-R-V-N-L-R-T-L-L-G-Y-Y.

When increasing amounts of this peptide were added to cells in culture, the synthetic Class I analogue significantly boosted the hormone binding on P815 mastocytoma cells in a dose-dependent fashion. Whereas the peptide was able to enhance receptor activity by at least an order of magnitude, peptides of similar size representing sequences from a T-cell receptor or MHC Class II sequences failed to augment the levels of hormone binding on P815. In addition, an H-2L<sup>d</sup> peptide synthesized without glutamic at position 71, but otherwise identical to L<sup>d</sup> 61-85, also was unable to affect insulin binding, demonstrating the exquisite specificity of this peptide for the receptor.

Several other cell types were tested for L<sup>d</sup> peptide enhancement of insulin binding, including freshly isolated murine spleen. (See Table 4) Cells of both human and murine origin demonstrated insulin binding augmentation to varying degrees by the L<sup>d</sup> peptide. The magnitude of induction on BALB/c spleen cells supports the in vivo effect of the peptide.

Table 4  
Effect of L<sup>d</sup> Peptide on Cell Types/Hormone Receptors

Cell Type/Line	Origin	Hormone	cpm <sup>125</sup> I-Insulin (% Bound/Free)		Relative Fold Increase with Peptide
			L <sup>d</sup> Peptide	No Peptide	
BALB/o Spleen	murine	Insulin	2,803 (6.0)	163 (.04)	150
P815 Mastocytoma	murine	Insulin	1,223 (2.0)	95 (.15)	13.3
Lymphoblastoid #1	human	Insulin	5,905 (13)	3,743 (8.0)	1.6
Lymphoblastoid #2	human	Insulin	2,409 (5.0)	1,306 (3.0)	1.7
LR 335 Fibrosarcoma	murine	EGF	2,394 (3.8)	2,543 (4.1)	0

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Insulin binding on various cell types in the presence and absence of Class H-2L<sup>d</sup> peptide. All cell lines are described above. Cells were pre-incubated with or without the L<sup>d</sup> peptide, aa<sup>61-85</sup> and specific <sup>125</sup>I-insulin binding was measured as described in Methods. When two non-Class I peptides, murine T-cell receptor and murine Class II peptide were used instead of the L<sup>d</sup> peptide, results were similar to no added peptide. Results are expressed as bound cpm of <sup>125</sup>I-insulin and the percent of counts Bound/Free <sup>125</sup>I-insulin.

Furthermore, no effect was observed when epidermal growth factor (EGF) binding was measured on a fibro-sarcoma expressing EGF receptors, showing further specificity for the peptide in interacting with insulin  
5 receptors.

Additional insulin binding studies were carried out on a series of related cells possessing different surface expression profiles of Class I/IR complexes. The insulin binding level on R1E,  
10 R1E+B2M+D<sup>b</sup> were tested in the presence and absence of L<sup>d</sup> peptide. The H-2L<sup>d</sup> peptide was capable of augmenting the IR found on the D<sup>b</sup> transfectants alone - both R1E and R1E+B2M+K<sup>b</sup> failed to show appreciable L<sup>d</sup> peptide induced enhancement in insulin binding.

15

Table 5  
Effect of L<sup>d</sup> Peptide on Insulin Binding  
on R1E, R1E+ $\beta_2$ M+D<sup>b</sup>, R1E+ $\beta_2$ M+K<sup>b</sup>

20

<u>Cell Line</u>	<u>cpm <sup>125</sup>I-Insulin (%B/F)</u>	
	<u>L<sup>d</sup> Peptide</u>	<u>No Peptide</u>
R1E	752 (1.0)	393 (1.0)
R1E + $\beta_2$ M + D <sup>b</sup>	935 (2.0)	471 (1.0)
25 R1E + $\beta_2$ M + K <sup>b</sup>	478 (1.0)	432 (1.0)

In agreement with the previous observations, the H-2K<sup>d</sup> transfectants appear to lack cell surface IR since no appreciable hormone binding could be induced  
30 with the peptide. Consequently, these data confirm that the D-end products control the transport of receptor to the cell surface.

Other studies were performed with 61-85 fragments of D<sup>k</sup> antigens, as well as the use of a  
35 number of control peptides. Insulin activity was segregated into two factors, tyrosine kinase activity and glucose uptake.

### Peptides

The two MHC Class I derived peptides D<sup>k</sup>-(61-85), and K<sup>k</sup>-(61-85) are both from the same region of the α1 domain of the MHC Class I molecules (Klein, Natural history of the major histocompatibility complex (Wiley, New York)). Both peptides were synthesized by Applied Biosystems, Inc., (Foster City, CA), and quality controlled by mass spectrometry.

The D<sup>k</sup>-(61-85) and K<sup>k</sup>-(61-85) peptides were iodinated for some experiments using carrier-free Na<sup>125</sup>I (Amersham) and iodobeads (Pierce) by incubating for 20 min., then purified by reversed-phase HPLC on a C<sub>18</sub> column (Beckman) in a linear 30-50% gradient of CH<sub>3</sub>CN in 5 mM trifluoroacetic acid (TFA). The <sup>125</sup>I-labeled peptide eluting first was stored at 4°C in 50% CH<sub>3</sub>CN/5 mM TFA. The labeled peptides were stable under these conditions for at least 3 months.

Control Peptides: ACTH-(1-24) (human), CCK-33 (porcine), dynorphin A (porcine), β-endorphin-(1-27) (camel), glucagon (human), and prosomatostatin-(1-32) (porcine) were all purchased from Peninsula Laboratories, Belmont, CA. The A-chain and B-chain of insulin (porcine) and glucagon-(1-21) (human) were obtained from Novo Industry, Denmark. ACTH-(1-24) was used as a routine control peptide.

### Purified Insulin Receptor

The purified human IR and the cloned cytoplasmic kinase domain (IRKD) have been described (Ellis *et al.* (1988) Virology 62:1634-39; Roth *et al.* (1986) J. Biol. Chem. 261:3753-57). Briefly, the human IR was purified from placenta by immunoaffinity columns, using monoclonal antibodies and binding of IR to wheat germ agglutinin. The product was a tetramer with two heavy chains, each ~130 kDa, and two light chains, each ~90 kDa.

### Tyrosine Kinase Activity

The cytoplasmic, cloned IRKD was constructed from the IR sequence (Ebena *et al.* (1985) *Cell* 40:747-758; Ullrich *et al.* (1985) *Nature* 313:756-761) and expressed in insect cells by using a baculovirus expression vector. The domain is soluble ( $M_r$  ~48 kDa) and the kinase activity is constitutively expressed *in vitro*. The IRKD was purified to homogeneity by immunoaffinity chromatography.

The procedures to measure kinase activity of the purified IR and IRKD, and the effects of insulin have been described elsewhere (Roth *et al.* (1986) *supra*). Briefly, 5.0  $\mu$ l purified IR was mixed with 5.0  $\mu$ l insulin (final concentration 1.0  $\mu$ M), and buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 0.1% Triton X-100) added to a final volume of 20  $\mu$ l. When peptide was used, it was added in 5.0  $\mu$ l, the volume adjusted to 20  $\mu$ l by adding buffer, and the mixture incubated (1 hr, 4°C). After incubation, 10  $\mu$ l of a solution containing 2.5  $\mu$ Ci  $^{32}$ P-labeled ATP (3,000 Ci/mmol;  $\gamma$ -labeled; Amersham), 50 mM HEPES, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 37.5  $\mu$ M unlabeled ATP, 15 mM  $MgCl_2$ , and 6 mM  $MnCl_2$  was added to a final volume of 30  $\mu$ l. The mixture was then incubated for 30 min. at 24°C.

After incubation, 15  $\mu$ l sample buffer was added, and the sample was boiled for 5 min., and run on 10% SDS-PAGE overnight. The gel was dried, and autoradiograms processed with an exposure time of 5-10 hr. For quantitative estimates the  $\beta$ -subunit band of IR and the IRKD bands were cut out and counted dry (Cerenkov) in a scintillation counter.

Substrate phosphorylation was done with poly-([Glu,Tyr];4:1) (Sigma) as substrate. The substrate was added to a final concentration of 1.0 mg/ml and the phosphorylation assay was conducted as described above. For quantitative estimates the entire lane from just

below the insulin receptor band to a 20-kDa marker was cut out and counted or the substrate was precipitated with TCA. For the latter, 5  $\mu$ l sample was dotted on to 3 MM paper (Whatman), washed 30 min. in ice cold 10% TCA, boiled 10 min. in 5% TCA, then washed twice in distilled water and twice in ethanol, and finally dried and counted.

#### Insulin Binding

10 Porcine monoiodinated [ $^{125}$ I]-insulin (iodinated at Tyr A14; 1,900-2,000 Ci/mmol) was obtained from NOVO Industry and Amersham. Unlabeled porcine insulin (NOVO) was dissolved in 10 mM HCl at 1 mM and stored immediately at  $-20^{\circ}\text{C}$ .

15 The plate assay for insulin binding to its purified receptor has been described (Morgan and Roth (1985) Endocrinology 116, 1224-1226). Briefly, 50  $\mu$ l of affinity-purified rabbit anti-mouse IgG (Jackson Immuno Research Lab., Inc., West Grove, PA) (40  $\mu\text{g}/\text{ml}$ )  
20 in 20 mM  $\text{NaHCO}_3$ , pH 9.6, was added to 96-well polyvinyl chloride (PVC) plates. The plates were incubated (17-20 hrs,  $4^{\circ}\text{C}$ ), washed thrice in 50 mM HEPES, pH 7.8, with 150 mM NaCl, 0.1% Triton X-100, 0.05% BSA, and  $2 \times 10^{-8}$  M monoclonal antibody (Amac, Inc., Westbrook, ME)  
25 was added. After incubation (1 hr,  $24^{\circ}\text{C}$ ), the plates were washed, and insulin binding measured.

For binding measurements,  $^{125}\text{I}$ -insulin ( $3 \times 10^{-10}\text{M}$ ) was added together with increasing amounts of unlabeled insulin, and incubated (90 min.,  $24^{\circ}\text{C}$ ),  
30 washed, and the amount of free and bound  $^{125}\text{I}$ -labeled insulin measured. Bound insulin was determined by eluting IR off the plate with 0.1M HCl and measuring in a  $\gamma$ -counter. For data analysis, non-specific binding was defined as the amount bound in presence of  $10^{-6}\text{M}$   
35 unlabeled insulin.



### Results

The effect of D<sup>k</sup>-(61-85) on both substrate (poly-[E,Y]) phosphorylation and IR autophosphorylation as a function of the peptide concentration, wherein IR tyrosine kinase activity is induced with 10<sup>-6</sup> M insulin was determined. Both are strongly inhibited at a concentration of μM D<sup>k</sup>-(61-85). The basal activity of IR (no insulin added) is inhibited 24-40% by D<sup>k</sup>-(61-85) and K<sup>k</sup>-(61-85). The effect of K<sup>k</sup>-(61-85) is significantly weaker than D<sup>k</sup>-(61-85) on autophosphorylation, with EC<sub>50</sub> values [95% confidence intervals] of 4.0 μM [2.2-7.2 μM] and 1.2 μM [0.3-2.2 μM] for K<sup>k</sup>-(61-85) and D<sup>k</sup>-(61-85), respectively, whereas no difference is observed in respect to substrate phosphorylation. None of the control peptides (e.g. ACTH-(1-24)) are substrates for IR tyrosine kinase.

No significant depletion (degradation or adsorption), as examined by HPLC and <sup>125</sup>I-labeled D<sup>k</sup>-(61-85), K<sup>k</sup>-(61-85), ACTH-(1-24), or dynorphin A is observed during the experimental period at concentrations above 0.1 μM. The D<sup>k</sup>-(61-85) peptide does not affect IRKD phosphorylation, as demonstrated by pre-incubation of maximally autophosphorylated and <sup>32</sup>P-labeled IR for 1 hr on ice with 10 μM peptide and subsequent incubation with 500 μM cold ATP for 0-60 min. at room temperature.

The D<sup>k</sup>-(61-85) has no effect on the binding of insulin to IR. The EC<sub>50</sub> IR autophosphorylation is about 3 x 10<sup>-9</sup> M insulin, corresponding approximately to K<sub>d</sub> (2.8 x 10<sup>-9</sup> M). D<sup>k</sup>-(61-85), at 10 μM inhibits autophosphorylation at all insulin concentrations.

D<sup>k</sup>-(61-85), 3 μM inhibits the insulin-induced IR autophosphorylation, but not the insulin receptor kinase domain phosphorylation, when IR and IRKD are used at comparable activities. IR is not a significant substrate for IRKD in the absence of insulin. IR becomes a significant substrate for IRKD when insulin

is added. This observation is facilitated by the inhibitory effect of the peptide on IR autophosphorylation, because the IR phosphorylation as mediated by the tyrosine kinase of IR itself and the phosphorylation mediated by IRKD would otherwise be indistinguishable.

In the next study, the uptake of glucose in rat adipocytes was performed. Adipocytes are prepared from non-starved male rat epididymal fat pads (1.2-1.6 g fat per rat) by collagenase digestion. The buffered is KRH with 5% BSA: only plastic tubes are used. The digest is filtered (25  $\mu$ l) washed and resuspended in approximately 4 x the cell volume (estimated by lipocrit). An aliquot is removed for Coulter counting after staining with 2% osmium tetroxide, filtration and dilution in saline. 50  $\mu$ l of adipocyte suspension is added to the pre-incubation mix; 300  $\mu$ l buffer, 50  $\mu$ l insulin (80 nM) or buffer; 50  $\mu$ l test solution (10 x) or buffer and incubated for 30 min. at 37°C in a shaking water bath. A blank without cells is included for background counting. D-[<sup>14</sup>C]-glucose is subsequently added (about 10<sup>5</sup>dpm/sample) and incubation continued for 60 min. The incubation is terminated by layering the 400  $\mu$ l sample on top of silicone oil, followed by a 30 sec. microfuge spin, and cutting the adipocytes (thin layer of cells on top of the oil, buffer under oil) into LS vials with scintillation fluid. Glucose concentration was about 300 nM (sp.a. 295 mCi/mmol).

The effect of increasing concentrations of insulin in 30  $\mu$ M D<sup>k</sup>-(61-85) on glucose uptake was determined. Insulin induced maximally and 8-11 fold increase in glucose uptake as compared to basal uptake. Addition of D<sup>k</sup>-(61-85) increased the maximal uptake to about 14-18 fold of basal, a glucose uptake above maximal insulin stimulation. At low concentrations of insulin (plasma level and lower), 30  $\mu$ M D<sup>k</sup>-(61-85)

increased glucose uptake as much or more than insulin on a molar basis.

The maximal effect of D<sup>k</sup>-(61-85) was obtained at 15  $\mu$ M. It is found that the increase varies with the particular peptide batch, where the insulin effect of the peptide may vary from about 20% to 100%.

Various fragments of D<sup>k</sup>-(61-85) were prepared by enzymatic digestion with specific peptidases: endo K, which gave fragments 61-68 and 69-85; endo E, which gave fragment 78-85; CP Y, which provided fragment 61-84; and in addition, the starting fragment was iodinated, which would be expected to occur at the terminal tyrosines. Each of the fragments were tested for biological activity after purification (greater than 95%) by HPLC and added to cells to a final concentration of 30  $\mu$ M. The results reported as percent activity of the mean  $\pm$  SE, with the starting fragment being 100 are as follows (61-68) 19  $\pm$  22; (69-85) 87  $\pm$  2; (78-85) 15  $\pm$  3; (61-84) 19  $\pm$  3; iodinated fragment 9  $\pm$  10.

The effect of D<sup>k</sup>-(61-85) in whole rats was determined. D<sup>k</sup>-(61-85) (2.5 mg/kg) and insulin (10  $\mu$ g/kg) on the blood glucose levels in rats (100-300 g) was determined. The peptide and insulin were injected i.v. after the animals had been anesthetized with pentobarbital. All animals were starved 16-20 hrs. prior to experimentation. Each determination was based on results as obtained from 42 rats, where the same rats were used in the four treatment schedules. The schedules were a control, a peptide by itself, insulin by itself, and insulin plus peptide. The control showed no significant change in blood glucose over the 240 min. during which determinations were made. The peptide, at about 20 min., the blood glucose had dropped to about 65% of its original value and then slowly rose back to about the original value at about 90 min. and was maintained about the same level. A

similar result was observed with the injection of insulin. However, where the insulin and peptide were injected together, the glucose dropped within about 20 min. to about 55% of its original value and slowly rose to about 85% of its original value at about 195 min. then gradually increase to about 90% at about 240 min. Calculation of the area between the control curve and the experimental curves from  $T = 0$  to  $T = 240$  showed that the area for insulin plus peptide is significantly larger than that of insulin or peptide alone, indicating a prolonged hypoglycemia period as compared to the insulin or peptide alone.

In order to determine the main target organs for peptide mediated glucose, the glucose uptake in various organs was analyzed as a function of oligopeptide injection i.v. The main target organs for peptide mediated glucose uptake are skeletal muscle, liver and kidney, when the size of the organ is considered. It is notable that some of these organs are not affected by insulin injection. The procedure employed was the injection of  $^{14}\text{C}$ -2-deoxyglucose 60 min. after injection of insulin plus peptide and the organ content of  $^{14}\text{C}$  measured 30 min. later, i.e. 90 min. after injection of peptide.

Based on the above data, it may be concluded that  $\text{D}^{\text{k}}-(61-85)$  peptide enhances cellular glucose uptake both in the absence and presence of insulin. Peptide effect is increased upon stimulation with insulin. Maximal peptide effect is reached at a peptide concentration of 10-20  $\mu\text{M}$ . The peptide causes enhanced glucose uptake significantly above that induced by maximal insulin stimulation. The effect in vitro is maximal after 20 min. incubation of the cells with peptide. Intravenous injection of 2.5 mg/kg  $\text{D}^{\text{k}}-(61-85)$  peptide causes a decrease in blood glucose in whole animals. It is accentuated when insulin is injected together with the peptide. Glucose uptake as

induced by peptide is particularly pronounced in muscle, liver and kidney, but the peptide does not result in increased levels of serum-insulin.

It is evident from the above results that  
5 surface membrane receptors involving transduction of signals, as exemplified by the insulin receptor, are modulated by MHC Class I antigens, particularly H-2D and -L of mice and HLA-B and -C of humans. A wide variety of physiological processes, both in vitro and  
10 in vivo, may be regulated by controlling the interaction between the appropriate Class I antigen and the surface membrane receptor, by a variety of techniques which allow for the enhancement or reduction of the interaction between the Class I antigen and the  
15 surface membrane receptor.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent  
20 applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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WHAT IS CLAIMED IS

1. A method for regulating surface receptor response of a mammalian cell, said method comprising:  
5 modulating the number of binding complexes or functionally equivalent binding complexes between (1) a major histocompatibility complex human Class I HLA-B or -C antigen or other mammalian equivalent thereof and (2) said surface receptor on  
10 said cell by varying the number of said antigens on the surface or adding a moiety capable of binding to said antigen or receptor to mimic binding complex formation or inhibit binding complex formation, whereby said receptor response is modulated.  
15
2. A method according to Claim 1, wherein said modulating is binding an oligopeptide mimicking the complexing site of said antigen with said surface receptor to form said functionally equivalent complex.  
20
3. A method according to Claim 1, wherein said modulating is contacting said cell with a drug which affects the number of said antigens on said cell.
- 25 4. A method according to Claim 1, wherein said modulating is binding an antibody to one of said antigen or surface receptor inhibiting formation of said complexes.
- 30 5. A method according to Claim 1, wherein said modulating is binding an oligopeptide to said antigen inhibiting the formation of said complexes.
- 35 6. A method according to Claim 1, wherein said receptor is internalised after binding to its ligand.

7. A method for regulating an endocrine surface receptor response of a human cell, said method comprising:

modulating the number of binding  
5 complexes or functionally equivalent binding complexes between (1) a major histocompatibility complex human Class I HLA-B or -C antigen and (2) said endocrine surface receptor on said cell by varying the number of  
10 said antigens on the surface or adding a moiety capable of binding to said antigen or receptor to mimic binding complex formation or inhibit binding complex formation, whereby said receptor response is modulated.

8. A method according to Claim 7, wherein  
15 said modulating is binding an oligopeptide of at least 6 amino acids to said surface receptor having substantially the same amino acid sequence as an amino acid sequence of the  $\alpha^1$  domain of an HLA-B or -C antigen.

20 9. A method according to Claim 8, wherein said sequence is within the sequence of the amino acid sequence from amino acid 55 to 90.

10. A method according to Claim 8, wherein  
25 said amino acid sequence of said oligopeptide is identical to an amino acid sequence of said antigen and is of at least about 8 amino acids and includes the sequence R Y Y.

30 11. A method for regulating an insulin receptor response of a mammalian cell, said method comprising:

modulating the number of binding  
complexes or functionally equivalent binding complexes  
35 between (1) a major histocompatibility complex human Class I HLA-B or -C antigen or mammalian equivalent thereof and (2) said insulin receptor on said cell by

varying the number of said antigens on the surface or adding a moiety capable of binding to said antigen or receptor to mimic binding complex formation or inhibit binding complex formation, whereby said receptor  
 5 response is modulated.

12. A method according to Claim 11, wherein said modulating is by contacting said insulin receptor with an oligopeptide of at least about 8 amino acids  
 10 having an amino acid sequence substantially the same as a sequence of the  $\alpha^1$  domain of said antigen.

13. A method according to Claim 12, wherein said sequence is within the the sequence of the amino  
 15 acid sequence from amino acid 55 to 90 and includes the sequence R Y Y.

14. A method according to Claim 13, wherein said amino acid sequence is the sequence from amino  
 20 acid 61 to 85.

15. An oligopeptide of at least 8 amino acids comprising a sequence of the  $\alpha^1$  domain of a major histocompatibility complex Class I HLA-B or -C antigen or  
 25 mammalian equivalent thereof consisting essentially of a sequence coming within one of the following sequence units:

- (a) D T aa<sup>32</sup> F V R F D S D aa<sup>40</sup> aa<sup>41</sup>
- (b) F V R F D S D aa<sup>40</sup> aa<sup>41</sup> S P R aa<sup>45</sup>
- 30 (c) W aa<sup>52</sup> E Q aa<sup>55</sup> aa<sup>56</sup> G P E Y W
- (d) W aa<sup>61</sup> aa<sup>62</sup> aa<sup>63</sup> T aa<sup>65</sup> aa<sup>66</sup> aa<sup>67</sup> K aa<sup>69</sup>  
 aa<sup>70</sup> aa<sup>71</sup> Q
- (e) W aa<sup>61</sup> aa<sup>62</sup> aa<sup>63</sup> aa<sup>64</sup> aa<sup>65</sup> aa<sup>66</sup> aa<sup>67</sup> K  
 aa<sup>69</sup> aa<sup>70</sup> aa<sup>71</sup> aa<sup>72</sup> aa<sup>73</sup> aa<sup>74</sup> aa<sup>75</sup> aa<sup>76</sup>  
 35 aa<sup>77</sup> aa<sup>78</sup> aa<sup>79</sup> aa<sup>80</sup> aa<sup>81</sup> aa<sup>82</sup> aa<sup>83</sup> aa<sup>84</sup>  
 aa<sup>85</sup>
- (f) E Q aa<sup>73</sup> aa<sup>74</sup> R V aa<sup>77</sup> aa<sup>78</sup> R aa<sup>80</sup> aa<sup>81</sup>



aa<sup>82</sup> R Y Y

wherein:

- 5           aa<sup>32</sup> is any neutral aliphatic amino acid of  
from 4 to 6 carbon atoms;  
          aa<sup>40</sup> is G, A, D or E;  
          aa<sup>41</sup> is G, A, D or E;  
          aa<sup>44</sup> is P, N, or Q,  
10           aa<sup>45</sup> is any aliphatic amino acid;  
          aa<sup>52</sup> is V, I, L or M;  
          aa<sup>55</sup> is any charged aliphatic amino acid;  
          aa<sup>56</sup> is a charged amino acid;  
          aa<sup>61</sup> is D or E;  
15           aa<sup>62</sup> is K, R, G or A;  
          aa<sup>63</sup> is any aliphatic amino acid other than  
basic of from 4 to 6 carbon atoms;  
          aa<sup>64</sup> is S, T or M;  
          aa<sup>65</sup> is any polar or basic amino acid of from  
20           4 to 6 carbon atoms;  
          aa<sup>66</sup> is any aliphatic amino acid of from 4 to  
6 carbon atoms;  
          aa<sup>67</sup> is any neutral aliphatic or aromatic  
amino acid;  
25           aa<sup>68</sup> is K or R;  
          aa<sup>69</sup> is any aliphatic neutral amino acid;  
          aa<sup>70</sup> is any aliphatic amino acid other than  
acidic of from 3 to 6 carbon atoms;  
          aa<sup>71</sup> is any aliphatic amino acid other than  
30           basic;  
          aa<sup>72</sup> is N or Q;  
          aa<sup>73</sup> is S, T, F, Y, H or W;  
          aa<sup>74</sup> is D, E, F, Y, H or W;  
          aa<sup>75</sup> is K or R;  
35           aa<sup>76</sup> is an aliphatic amino acid other than  
basic of from 4 to 6 carbon atoms;  
          aa<sup>77</sup> is a polar aliphatic amino acid of from 3

to 6 carbon atoms;  
aa<sup>78</sup> is a neutral aliphatic amino acid of from  
5 to 6 carbon atoms;  
aa<sup>79</sup> is K or R;  
5 aa<sup>80</sup> is a neutral aliphatic amino acid of from  
3 to 6 carbon atoms;  
aa<sup>81</sup> is a neutral aliphatic non-polar amino  
acid;  
aa<sup>82</sup> is an aliphatic amino acid other than  
10 acidic;  
aa<sup>83</sup> is an aliphatic amino acid other than  
acidic;  
aa<sup>84</sup> and aa<sup>85</sup> are aromatic amino acids;  
with the proviso that there are not more than  
15 three amino acid mutations as deletions, insertions or  
substitutions.

16. An oligopeptide according to Claim 15,  
wherein said oligopeptide sequence comes within the  
20 following unit:  
W D/E R aa<sup>63</sup> T Q/R aa<sup>66</sup> aa<sup>67</sup> K aa<sup>69</sup> aa<sup>70</sup> aa<sup>71</sup>  
Q T/W aa<sup>74</sup> R V/E aa<sup>77</sup> L R aa<sup>80</sup> L/A L/R G/R Y Y.  
where aa/aa intends either amino acid may be  
present.

25  
17. A peptide conjugate comprising an oligo-  
peptide according to Claim 15 covalently bonded to at  
least one amino acid, wherein said amino acid is other  
than the wild type amino acid.

30  
18. A peptide conjugate according to Claim  
17, wherein said at least one amino acid is an immuno-  
genic polypeptide capable of stimulating an immune  
response in a vertebrate host.

35.

19. A method for detecting the presence of an MHC Class I antigen dependent receptor present on a cell surface or at least a portion of a lysate, said method comprising:

5 combining a sample suspected of containing said receptor with a peptide conjugate according to Claim 16; and

determining the presence of a complex between said peptide conjugate and said receptor.  
10

20. A method for detecting the presence of an MHC Class I antigen dependent receptor present on a cell surface or at least a portion of a lysate, said method comprising:

15 combining a sample suspected of containing said receptor with an oligopeptide according to Claim 15; and

determining the presence of a complex between said oligopeptide and said receptor.  
20

21. An oligopeptide according to Claim 15 bonded to a label capable of providing directly or indirectly a detectable signal.

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## AMENDED CLAIMS

[received by the International Bureau on 10 April 1990 (10.04.90);  
original claim 15 amended ; other claims unchanged ( 1 page)]

to 6 carbon atoms;

5           aa<sup>78</sup> is a neutral aliphatic amino acid of from  
5 to 6 carbon atoms;

aa<sup>79</sup> is K or R;

aa<sup>80</sup> is a neutral aliphatic amino acid of from  
3 to 6 carbon atoms;

10           aa<sup>81</sup> is a neutral aliphatic non-polar amino  
acid;

aa<sup>82</sup> is a aliphatic amino acid other than  
acidic;

aa<sup>83</sup> is an aliphatic amino acid other than  
acidic;

15           aa<sup>84</sup> and aa<sup>85</sup> are aromatic amino acids;

with the proviso that there are not more than  
three amino acid mutations as deletions, insertions or  
substitutions.

20           16. An oligopeptide according to Claim 15,  
wherein said oligopeptide sequence comes within the  
following unit:

W D/E R aa<sup>63</sup> T Q/R aa<sup>66</sup> aa<sup>67</sup> K aa<sup>69</sup> aa<sup>70</sup> aa<sup>71</sup>  
Q T/W aa<sup>74</sup> R V/E aa<sup>77</sup> L R aa<sup>80</sup> L/A L/R G/R Y Y.

25           where aa/aa intends either amino acid may be  
present.

30           17. A peptide conjugate comprising an oligo-  
peptide according to Claim 15 covalently bonded to at  
least one amino acid, wherein said amino acid is other  
than the wild type amino acid.

35           18. A peptide conjugate according to Claim  
17, wherein said at least one amino acid is an immuno-  
genic polypeptide capable of stimulating an immune  
response in a vertebrate host.

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US89/00876

<b>I. CLASSIFICATION F SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4) C07K 7/06, 10; A61K 37/02 ; C07K 7/08 US CL. 530/324-328; 514/12-16						
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border: 1px solid black;">Classification System</th> <th style="border: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: middle;">US</td> <td style="border: 1px solid black;">530/324; 530/325; 530/326; 530/327, 530/328; 514/12; 514/13; 514/14; 514/15; 514/16</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	US	530/324; 530/325; 530/326; 530/327, 530/328; 514/12; 514/13; 514/14; 514/15; 514/16
Classification System	Classification Symbols					
US	530/324; 530/325; 530/326; 530/327, 530/328; 514/12; 514/13; 514/14; 514/15; 514/16					
<b>CHEMICAL ABSTRACTS</b>						
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>						
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>				
X	Bhagirath SINGH, <u>The Journal of Immunology</u> , 137, No. 7, 1986 "Alloantigenic Sites on Class I Major Histocompatibility complex antigens: 61-69 Region in the First Domain of the H-2k Molecule Induces Specific Anti-body and T cell responses", See the abstract on page 2311.	15-18				
X	Alison FINNEGEN, <u>Journal of Experimental Medicine</u> , 164, 1986 "The T Cell Repertoire For Recognition of A Phylogenetically Distant Protein Antigen" See Table IV and The Discussion on pages 905-908.	15-18				
A	Morten Simonsen, <u>Progress In Allergy</u> , 36, 1985 "Compound Receptors In Cell Membrane: Rumination from the Borderland of Immunology and Physiology" pages 151-176.	1-21				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"d" document member of the same patent family</p> </div> </div>						
<b>IV. CERTIFICATION</b>						
Date of the Actual Completion of the International Search <div style="text-align: center; font-size: 1.2em;">15 August 1989</div>		Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.2em; border: 1px solid black; padding: 2px;">07 FEB 1990</div>				
International Searching Authority <div style="text-align: center;">ISA/US</div>		Signature of Authorized Officer <div style="text-align: center;">             Fatemeh Moezie         </div>				